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Production of a Suitable Antibody and an Enzyme Immunoassay Kit for the Field Detection of T-2 Toxin

Annual Summary Report

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An enzyme-linked immunosorbent assay was developed and used to assay serum from immunized mice for T-2 specific antibody. The assay allows for direct measurement of T-2 antibody, with the capability for competitive inhibition studies using T-2 standard solutions.

Two objectives were specified for this study. They were to produce antibodies specific for T-2 toxin, and to demonstrate an immune enhancement, with respect to T-2 antibody production, from the vesicle-based antigen delivery method. The vesicle delivered antigen does produce T-2 specific antibodies to T-2 toxin. Potential benefit of using the vesicle-based

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SUMMARY

Phospholipid vesicles, liposomes, were used as an immune enhancement (adjuvant) medium to produce antibodies specific for T-2 toxin. Vesicles were prepared such that neat T-2 toxin and a solution of T-2 succinyl bovine serum albumin were each either encapsulated or adsorbed on vesicles. Permutations of these formulations were used to immunize mice by repeated subcutaneous injections.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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STATEMENT OF PROBLEM - INTRODUCTION

T-2 toxin belongs to the group of 12,13-epoxy-trichothecenes produced by several species of fungi including Fusarium and Tricoderma (1). Mycotoxins, such as T-2, are known to naturally occur on plants including food products. These trichothecenes are highly toxic to eukaryotic cells and are among the most potent low molecular weight inhibitors of protein and DNA synthesis (2-4).

Potential intoxication of humans and animals by T-2 toxin establishes the need for a simple and rapid method for the detection of T-2 toxin in the environment, and in food and biological samples. Present methods of detection require bioassays, which are time consuming and imprecise, or sophisticated instrumental analyses such as high performance liquid chromatography, gas chromatography or mass spectrometry. None of these analytical methods satisfy the requirements for a rapid and simple detection method which can be routinely run in a modestly equipped field laboratory.

An immunoassay procedure using antibodies specific for T-2 toxin would satisfy the criteria for a rapid and simple detection method. However, attempts to produce antibodies to T-2 toxin have encountered several problems. The low molecular weight T-2 toxin is not antigenic. Attempts to attach T-2 to an immunogenic carrier have successfully produced only one T-2 conjugate, T-2 succinyl-bovine serum albumin (T-2-BSA). Conventional immunization methods using the T-2-BSA conjugate have not produced satisfactory low-titer and low-affinity antibodies to T-2 toxin.

Recent work has shown that phospholipid vesicles (liposomes) can enhance immune responses to small antigens (5). Phase I of this project proposes to evaluate the adjuvancy potential of a variety of phospholipid vesicle formulations used in association with T-2 and T-2-conjugate.

BACKGROUND

The chemistry, toxicological and biochemical effects of T-2 toxin have been reported in detail (1). Chemical and instrumental methods for T-2 toxin detection and analysis have also been reviewed (6,7). The level of effort required and the sensitivity of detection has been reported for high performance liquid chromatography (8) and gas chromatography-mass spectrometry.

Recently, efforts have focused on developing an immunoassay method for the detection of T-2 toxin. The synthesis of radiolabeled T-2 toxin was reported by Wallace et al.(10). The (^{3}H) T-2 toxin was used by Chu et al. (11) to synthesize T-2 succinyl-bovine serum albumin conjugate as an antigenic conjugate for T-2 toxin antibody production in rabbits. Subsequently, the T-2-BSA conjugate has been used by several groups to produce anti-T-2 antibody for use in radioimmunoassays (RIA) (14, 15) for the detection of T-2.

A suitable means for routinely producing T-2 antibodies with high binding affinities and high titers is not yet available. T-2 antibodies with high specificity for T-2 toxin and high binding affinities are prerequisite for successful development of an enzyme-linked immunosorbent assay (ELISA) and a combination of high affinity, high titer antibodies are necessary for successful application of immunoassay techniques to portable, field detection methods for T-2

toxin.

Phospholipid vesicles have been used as adjuvants for antigens or haptens either alone with the vesicles or conjugated to immunogenic carriers (16, 17). Vesicle adjuvants have been shown to enhance immunogenic response to nonantigenic or weakly antigenic material (18-20). This response has resulted in the production of both IgM and IgG antibodies (21, 22).

The research objectives of Phase I of this contract were: to define the optimal vesicle-antigen system for stimulating production of T-2 antibodies; and to determine what effects permutations of vesicle-antigen encapsulation or linkage have on immune response. Permutations to be tested included:

1. The physical configuration of the vesicle-antigen complex;
2. The addition of adducts to vesicle-antigen formulations;
3. Variations of vesicle membrane composition; and
4. The route by which the vesicle-antigen complex is injected and the size of the vesicle used.

APPROACH TO PROBLEM - METHODS

Materials

T-2 toxin was supplied by the U. S. Army Medical Research Institute of Infectious Diseases. Tritium-labeled T-2 was prepared according to the method of Wallace et al. (10). The (^{3}H)T-2 was mixed with unlabeled T-2 toxin and used at a specific radioactivity of 50 mCi/mole for the studies described in this report.

Bovine serum albumin, Pentex, crystallized, was purchased from Miles. N,N-dimethylformamide, silylation grade, was obtained from Pierce Chemicals. Dicyclohexylcarbodiimide was obtained from Vega Biochemicals. All other organic reagents were obtained from Aldrich Chemical. Organic solvents were from Mallinckrodt Chemicals. The phospholipids and other vesicle components were purchased from Calbiochem and Sigma. Plates for thin layer chromatography were E. M. Merck Silica Gel 60. Aquasol scintillation cocktail was from New England Nuclear.

The animals used in this project were Swiss Webster female mice obtained from Simonsen's Laboratories Inc., Gilroy, CA. The mice generally weighed 20 gm at the start of immunization. Caging of animals and animal room facilities were in accord with NIH publication No. 80-23, Guide for the Care and Use of Laboratory Animals.

Preparation of T-2 Toxin Conjugate

The preparation of T-2 conjugate to BSA, as reported by Chu et al. (11), utilized water-soluble carbodiimide as the coupling agent. The carbodiimide reaction, which forms amides from amines and carboxylic acid group, will couple the carboxylic acid on the T-2 hemisuccinate to primary amines on BSA. However, the water soluble carbodiimide also couples carboxylic acid groups on BSA to amines on other BSA molecules. Thus, this method forms large multimers of BSA with T-2 also attached. These large BSA multimers become increasingly insoluble with size. Immunizations with this T-2 BSA conjugate generally involved injecting suspensions

of the antigenic material.

An antigen conjugate suitable for vesicle encapsulation must be soluble in aqueous solutions. Therefore, a preliminary task undertaken in Phase I was to synthesize a new, water-soluble protein conjugate of T-2 toxin.

The first method attempted to react the C-3 hydroxyl of T-2 with p-nitrophenyl chloroformate. Although the p-nitrophenyl-formyl-T-2 intermediate was synthesized, the subsequent reaction with BSA did not work and this synthesis was not pursued.

The second synthetic method used to produce a soluble T-2-BSA conjugate started with the production of T-2 hemisuccinate. T-2 toxin was reacted with succinic anhydride in anhydrous pyridine in the presence of 4-N,N-dimenthylaminopyridine. The intermediate T-2-hemisuccinate was then reacted with N-hydroxysuccinimide and dicyclohexylcarbodiimide to produce the activated succinimide ester. This reaction was performed in N,N-dimethylformamide (DMF). The activated T-2-succinimide in DMS was added to a solution of BSA in 0.1 M sodium bicarbonate buffer pH 8.2.

After 30 min., the reaction solution was adjusted to pH 6-7, using HCl, and the solution diluted with two volumes of water. Unreacted T-2-hemisuccinate was removed by gentle extraction with dichloromethane. The agueous solution was dialyzed against ultrapure water and the T-2-succinyl-BSA then recovered by lyophilization.

Since the activated succinimide ester is hydrolyzed in water, it was found that only a small fraction of the T-2-hemisuccinate actually attached to BSA. To insure a substitution ratio of six T-2 molecules per BSA, the conjugation reaction was run using a ratio of activated T-2 hemisuccinate to BSA of 100:1.

Vesicle Preparation/Encapsulation of T-2-BSA

The principal procedure used for encapsulating T-2-BSA conjugate was the production of small unilamellar vesicles (SUV) by probe sonication. In a typical preparation T-2-BSA, 20 mg, was dissolved in 2 ml phosphate-buffered saline (PBS) by gentle vortexing overnight. The T-2-BSA in PBS was then added to 70 mg of a distearoyl-L-α-phosphatidylcholine (DSPC):Cholesterol (Chol) mixture (mole ratio 2:1) and hydrated by agitation for 30 min. at 35-40°C. The mixture was then probe-sonicated for 30 min. at 35°C. The resulting SUV preparation was cleared of particulate matter by centriguation at 1600 x g for 10 min.

At this stage, three different treatments were followed to prepare an injectable preparation of T-2-BSA encapsulated in SUV vesicles. The initial treatment involved diluting the supernatant fraction to obtain various doses of T-2-BSA for injection. No attempt was made to separate free T-2-BSA from vesicle-encapsulated conjugate.

The second treatment isolated a vesicle fraction by passing the supernatant from the post-sonication centrifugation through a Sephacryl-300 column. The vesicle fraction eluted from the column was identified visually, by light-scattering measurements and by (^{3}H)T-2 radioactivity eluting in the void volume. Unencapsulated T-2-BSA was recovered by monitoring radioactivity eluting after the void volume.

A third treatment protocol followed the procedure for isolating a vesicle fraction, but included a trypsin treatment to remove T-2-BSA attached to the outside of vesicles. The vesicle fraction which eluted from the Sephacryl-300 column was made 0.05% (w/v) trypsin and incubated for 15 min. at room temperature. The trypsin-treated suspension was then passed through a second Sephacryl-300 column and the vesicle fraction separated from trypsin and free T-2 toxin.

After each vesicle preparation and treatment, the vesicle suspension was assayed for T-2-BSA concentration by scintillation counting of an aliquot of the suspension. The specific radioactivity of the starting T-2-BSA was used to determine the concentration in the assayed sample. The T-2-BSA concentration in the vesicle suspensions were then adjusted by diluting the suspensions with PBS prior to the injection described below.

Other methods of vesicle preparation examined in this study were the preparation of large unilamellar vesicles (LUV) by the reverse phase evaporation method of Szoka and Paphadjopoulos (23), and by a modified version of the syringe injection of phospholipid in alcohol into aqueous medium, as described by Fraley et al. (24). Briefly, using the REV method, a solution of T-2-BSA was added to a mixture of phospholipid in chloroform, and the organic solvent removed by evaporation under vacuum. The vesicles were then passed through a Sephadex G-50 column to remove solvent residue and encapsulated material.

Preparation of vesicles by the alcohol injection method was accomplished by pumping a solution of phospholipid in dioxane:methanol (2:1) through a 23 Ga. needle into a solution of T-2-BSA contained in a dialysis bag. The T-2-BSA solution was dialyzed against PBS during the phospholipid addition and for 2-3 hrs after vesicle formation was complete. The vesicle suspension was then recovered, centrifuged at low speed and passed through a gel permeation column as described above.

Immunization Methods

Several immunization protocols were tried to identify preferred routes of injection, optimal dosages of vesicle T-2-BSA and the most effective formulation of vesicle-encapsulated T-2-BSA. These will be described below and identified in the results section.

Protocol 1:

Four groups of mice were used for immunization. Group 1 mice received doses of 12, 18 and 25 µg T-2 equivalent of a mixture of vesicle encapsulated and unencapsulated T-2-BSA conjugate. Two mice were injected per dose of the SUV vesicles prepared according to the first treatment described above. The injections were administered subcutaneous at the base of the tail, in the vicinity of the perianal-lymph nodes.

Group 2 mice received analogous, i.e. 12, 18, 25 µg T-2 equivalent, injections of nonvesicle, free T-2-BSA. Two mice were injected with each dose and route of administration was the same as for Group 1.

Group 3 mice received higher doses of the same vesicle encapsulated and free T-2-BSA mixture used for Group 1. Doses of 12.5, 25 and 50 µg T-2 equivalent were given by a single subcutaneous injection in the back.

Group 4 mice received 12.5, 25 and 50 µg T-2 equivalent doses of nonvesicle, free T-2-BSA conjugate. For each dose two mice were given a single subcutaneous injection in the back.

Mice in each group were given booster injections of the same dose, formulation and route of administration. Freshly prepared booster injections were given at 1, 3 and 8 weeks after the start of immunizations. Blood samples were collected at 2, 3, 4 and 7 weeks, and the animals were bled-out 9 weeks after the start of immunizations.

Blood was collected by retrobulbar puncture using heparinized microcapillary tubes. The collected samples were centrifuged, and plasma collected, pooled by group and stored at -20°C until assayed for T-2 antibodies.

Protocol 2:

Mice were immunized with a formulation in which T-2-BSA was totally vesicle-associated, either as encapsulated conjugate or conjugate firmly attached to the vesicle surface. Vesicles containing DSPC:Chol:DCP were made as described for the second treatment method for vesicle preparation.

Four groups of 10 mice were immunized with the vesicle-associated conjugate. Two groups received a one-time only injection. One of these groups received 6 µg T-2 equivalent by subcutaneous injection in the back. The other group received 13 µg T-2 equivalent. The remaining two groups also received the 6 and 12 µg doses, but these mice were given booster injections at 3, 4, 5 and 6 weeks after the initial immunization.

Animals in all four groups were bled at weeks 2, 4, 5, 7 and 8. Plasma was isolated and pooled according to groups.

Protocol 3:

In a third immunization scheme, mice were injected with either vesicle encapsulated T-2 toxin, vesicle encapsulated T-2-BSA conjugate or a free T-2-BSA conjugate. SUV vesicles containing T-2 toxin were prepared by drying a solution of T-2 toxin with the vesicle phospholipid. The dried film was then hydrated with PBS, vesicles prepared by sonication and a vesicle T-2 fraction isolated according to the second treatment method described in the vesicle preparation section.

SUV vesicles containing entrapped T-2-BSA were prepared according to the third method described in the section on vesicle preparation. The concentration of T-2-BSA in the vesicle preparation was calculated based on measurements of (^{3}H)T-2-BSA in the vesicle fraction. A solution of the free T-2-BSA conjugate was prepared in PBS and the concentration of T-2-BSA adjusted to equal the concentration in the vesicle T-2-BSA preparation.

Subsequently, mice were immunized with 40 µg T-2 equivalent in the form of T-2 encapsulated in vesicles, T-2-BSA encapsulated in vesicle, and a solution of free T-2-BSA. Booster injections using the same 40 µg dose were given on weeks 2, 3 and 4 following the initial immunization. Blood samples were collected from animals in each treatment group on weeks 1, 2, 3 and 4.

Antibody Assay

Plasma from immunized mice was assayed for anti-T-2 and anti-T-2-hemisuccinate antibodies by an enzyme-linked immunosorbent assay (ELISA). Serial dilutions of plasma were incubated in microtiter plates coated with T-2-succinyl-ovalbumin or T-2 toxin, for assays to detect T-2 antibodies, and BSA or ovalbumin, for assays to detect cross-reactive antibodies. The plates were then sequentially washed and incubated with rabbit anti-mouse IgG, and goat anti-rabbit IgG conjugated with alkaline phosphatase. The adherent antibody was then measured colorimetrically (OD_{400}) after the addition of the enzyme substrate p-nitrophenyl phosphate. The amount of anti-T-2 or anti-T-2-hemisuccinate present in the serum was calculated from a standard curve which correlated OD_{400} with known amounts of a reference antibody (µg/ml).

RESULTS

Vesicle Preparation/Encapsulation of T-2 and T-2-BSA

The interaction of T-2 toxin and the T-2-BSA conjugate with vesicle phospholipid greatly limited the number of permutations of vesicle composition and vesicle adducts tested in this study. Addition of a high mole ratio of cholesterol or a cholesterol derivative, such as aminomannosyl cholesterol, reduced the vesicle encapsulation efficiency of T-2 toxin and the T-2-BSA conjugate. Reduction of cholesterol mole ratio and/or addition of dicetylphosphate increased the encapsulation efficiency of T-2 and T-2-BSA.

Neat T-2 toxin was encapsulated in DSPC vesicles with nearly 30% encapsulation efficiency. The resultant vesicle preparation contained 5% (w/w) T-2:vesicle lipid. Likewise T-2 conjugated to BSA increased the amount of BSA encapsulated. Typically, vesicle encapsulation of BSA results in entrapment of 10-12% of the starting material. When the T-2-BSA conjugate was used 25-30% of the starting material was encapsulated. The significance of this observation will be addressed further in the discussion and recommendation.

Toxicity of Vesicle T-2 Toxin

Vesicle encapsulated neat T-2 toxin was used to establish an approximate toxic dose for vesicle T-2. Mice were injected subcutaneously with free T-2 in ethanol, PBS or with vesicle-T-2 in PBS. The LD_{50} for a 20 gm mouse was taken to be 40 µg. As shown in Table I mice given vesicle T-2 at the LD_{50} dose for the free toxin survived. Mice given vesicle T-2 at two and three times the LD_{50} survived 5-6 times longer than the mice given the neat T-2.

Immunizations with Vesicle T-2-BSA

Assays for T-2 antibody production in mice immunized according to the protocols described above are incomplete. Plasma samples from mice immunized with a mix of vesicle encapsulated and free T-2-BSA (protocol 1) have been assayed. Plasma samples collected at week 4 of immunization were assayed for T-2 antibody and crossreactivity of plasma antibodies with BSA and ovalbumin. The results of the crossreactivity assays are present in Table II.

TABLE I

T-2-TOXICITY

SURVIVORS/TOTAL NUMBER TREATED - (SUBCUTANEOUS)

	DOSE (X LD_{50})				
TREATMENT	0.10	0.25	1.0	2.0	3.0
Free T2	3/3	3/3	0/3 (24 HR)	0/3 (12 HR)	0/3 (12 HR)
Vesicle W/T2 Associated	NA	NA	3/3	0/3 (72 HR)	1/3 (72 HR)
Control	3/3		3/3		3/3

Survivors kept for one month.

Times in parentheses are time to last observed death.

Survivor weights and weight gains were similar except for Free T2 (0.25 LD_{50}).

TABLE II

CROSSREACTIVITY ASSAYS OF 4-WEEK ANTISERA

PLATE COATING	ANTISERUM TESTED	ELISA TITER (µg/ml-IgG STANDARD)
BSA	Anti-BSA	6770
BSA	Free T2-BSA	1700 ± 120
BSA	Vesicle T2-BSA	1750 ± 40
Ovalbumin	Anti-BSA	170
Ovalbumin	Free T2-BSA	0
Ovalbumin	Vesicle T2-BSA	0
T2-Ovalbumin	Anti-BSA	6.5 ± 0.4
T2-Ovalbumin	Free T2-BSA	403 ± 7.0
T2-Ovalbumin	Vesicle T2-Bsa	1020 ± 120

Anti-BSA antibody was a purified preparation supplied by Dr. J. David Beatty, City of Hope. Free T-2-BSA antiserum was a 4 week sample collected and pooled from group 1 mice described in protocol 1. Vesicle T-2-BSA antiserum was collected and pooled from group 2 mice in the same study.

As expected immunizing with free or vesicle encapsulated T-2-BSA produced anti-BSA antibodies. This is indicated by the ELISA titers obtained with BSA-coated plates (Table II). The ovalbumin and T-2-ovalbumin coated plates were run to detect crossreactivity of the anti-BSA antibody and the presence of antibodies specific for the T-2-conjugate. The results given in Table II indicate that anti-BSA antibodies do not crossreact with ovalbumin or T-2-ovalbumin. Table II also contains evidence that both antiserum from vesicle-T-2-BSA immunized mice and mice immunized with free T-2-BSA contained antibodies recognizing T-2-conjugated to protein.

Plasma samples collected weekly from each of the four groups of mice in protocol 1 were assayed on T-2-ovalbumin coated plates. Anti-T-2 antibodies were produced in each of the groups (Table III). Antibody titers at four weeks were suitably high for all groups except for the mice receiving the high dose of free T-2-BSA-conjugate. An explanation for the similar responses with the low dose of free T-2-BSA and the vesicle T-2-BSA was produced after analysis of the vesicle T-2-BSA formulation. Nearly all the T-2-BSA in the DSPC:Chol vesicles prepared according to the first vesicle treatment procedure was weakly associated with vesicles. Chromatographic isolation of vesicles resulted in separation of vesicles from T-2-BSA. Hence, this immunization protocol was comparable to conventional immunization formulations and did not differ greatly from injections of the free conjugate.

TABLE III

ANTI-T2 ELISA SUMMARY

	ELISA TITER (μg/ml-IgG STANDARD)		
TREATMENT	2 Wks	3Wks	4 Wks
Vesicle T2-BSA (High Dose)	119 ± 9	66 ± 2	918 ± 44
Vesicle T2-BSA (Low Dose)	105 ± 2	85 ± 11	663 ± 77
Free T2-BSA (High Dose)	34 ± 0.2	63 ± 0.7	284 ± 9
Free T2-BSA (Low Dose)	99 ± 0.2	120 ± 4	838 ± 21

TABLE IV

ANTI-T2 ELISA SUMMARY

TREATMENT	ELISA TITER (μg/ml) 2 Wks	4 Wks
Vesicle T2-BSA (High Dose - 250 T2-BSA/Injection)	3.4 ± 0.03	162 ± 16
Vesicle T2-BSA (Low Dose - 125 T2-BSA/Injection)	1.9 ± 0.1	102 ± 4

Preliminary results from immunizations with isolated DSPC:Chol:DCP vesicles with encapsulated and surface-bound T-2-BSA are given in Table IV. Antibody assays were again run on T-2-ovalbumin coated plates. The results demonstrate that vesicle-associated antigen produced antibodies to the T-2-conjugate.

Following discussions with USAMRIID personnel, a decision was made to modify the ELISA assay so that the antibody specificity would be better defined. The goal was to demonstrate antibody specificity to T-2 toxin. This required elimination of any possibility that the determinant recognized in the ELISA was the T-2-succinyl-lysine moiety common to T-2-BSA, T-2-ovalbumin and T-2-poly-lysine.

ELISA plates were coated with free T-2 toxin and the antisera from protocol 1 reassayed in a similar fashion as previously described. Anti-T-2 titers measured using the T-2 coated plates were approximately 2% of the titers observed with the T-2-ovalbumin coated plates.

Competition assays were also run in which test antisera was treated with a standard solution of free T-2 prior to ELISA-type assays run on T-2 and T-2-ovalbumin coated plates. Competition with free T-2 effectively reduced apparent antibody levels on T-2 coated plates but did not result in a detectable change in absorbance on T-2-ovalbumin plates. This is consistent with the approximately 2% T-2 specificity in the T-2-ovalbumin assay. The ELISA using T-2 coated plates is currently being used to assay samples from the protocols described above.

DISCUSSION

Phospholipid vesicle encapsulation of T-2-succinyl-bovine serum albumin and subsequent subcutaneous injection in mice produces antibodies which specifically recognize T-2 toxin. The presence of T-2 specific antibody was demonstrated in ELISA-type assays with pure T-2 toxin as the coated antigen. The positive direct ELISA assay could be inhibited by competition with a standard solution of pure T-2 toxin.

However, the titers for T-2 specific antibodies are low, 2-4 µg/ml, in the plasma samples tested thus far. Several factors may be responsible for the low titer. First, it is known that T-2 toxin is a weak antigen. Therefore repeated immunizations and longer immune response times may be required to obtain high titers. This study has already shown that vesicle encapsulation T-2-BSA can be repeatedly injected at high doses without acute toxic consequences. Antibody production from an immunization protocol following this rationale is currently being run.

Secondly, the behavior of both free and BSA conjugated T-2 is very hydrophobic. T-2 and T-2-BSA in vesicles probably involves the T-2 molecule inserting into the phospholipid membrane. Thus, the T-2 is not optimally presented to antibody-producing cells. Increasing the hydrophilic properties of the T-2 molecule without dramatically altering its chemical structure would likely result in increasing antibody production. This can be achieved by at least two methods.

A T-2 toxin conjugate having a hydrophilic linker group would allow better presentation of the T-2 molecule during vesicle interaction with immune cells. T-2 formyl, T-2 tartaryl and T-2 caproyl linkages to phosphatidylethanolamine or to BSA fragments, and subsequent encapsulation or attachment to vesicles, are currently being synthesized or planned for synthesis.

Initially, the synthesis of the above conjugates will involve production of the active intermediate complex T-2-p-nitrophenyl formate. This reaction is very rapid and produces high yield. Although this reaction scheme was tried early in Phase I and found unsatisfactory for producing the BSA conjugate, the stereochemical hinderances associated with linking to intact BSA are not expected and reaction conditions can be more favorable for conjugate production since the restraints of maintaining a native protein conformation do not apply.

A second method for improving T-2 antibody production would form a polar derivative of T-2 toxin. Several candidate reagents are available which would cause a rearrangement of the T-2 epoxide following reaction across the 9,10-position double bond. The hydrophilic derivative could then be used with vesicle adjuvants for antibody production.

Immunizations following this rationale would require modification of immunoassay methods using the resulting antibodies. In all likelihood the antibody specificity would be directed toward the T-2 derivative. This would require that assays for T-2 in test samples would have to incorporate the same derivatization step prior to immunoassay.

Another discovery of the Phase I effort has been the apparent lypophylic character of T-2 toxin. As indicated in the results, neat T-2 readily associates with phospholipid vesicles. This presents two new routes for vesicle application for T-2 detection.

In addition to the observed T-2 association with vesicles, other research activities at Vestar have discovered ways of attaching similar vesicle formulations to glass and plastic surfaces. The direct ELISA used in the Phase I studies showed that plating with free T-2 was possible and advantageous. However, T-2 attached to the surface of vesicles may provide an improved immunoassay method. Two <u>in vitro</u> applications are possible.

Vesicle T-2 could function as an immunosorbent medium for isolating T-2 specific antibody. An early test of this possibility would involve coating ELISA plates with vesicle T-2. Test antisera would then be assayed with and without prior treatment with a standard solution of free T-2. The results would indicate whether antibody recognition is unaltered by T-2 association with vesicles.

If favorable results are obtained, the vesicle T-2 phenomenon could be used to isolate and concentrate T-2 toxin from test samples. Following initial treatment steps the test solution would be incubated with a vesicle solution. The vesicle fraction could then be isolated and plated directly on ELISA plates for subsequent assay.

Recommendations for Phase II

In light of Phase I results in which T-2 coated ELISA plates have given a positive assay and only low T-2 specific titers detected in the antisera tested, several additional tasks are proposed for the Phase II effort. These added tasks are: completion of antibody screening in antisera samples from all immunizations and later collection and screening of antiserum samples from the mice previously immunized; development of a T-2 immunoassay using T-2 vesicles as the coating antigen medium; and synthesis of new T-2 conjugates with hydrophilic linkers and vesicle attachment groups.

Assays of antisera samples from Phase I immunizations is continuing. However, production of T-2 specific antibodies appears to be a delayed response even though some immune enhancement with vesicles was observed. Animals which produced T-2 specific antibodies may respond to reimmunization in Phase II. Current plans are to reimmunize these animals after 4-6 weeks and check for increased antibody production.

T-2 toxin attached to phospholipid vesicles may be a reproducible method for coating ELISA plates for antibody screening. The vesicle absorption of T-2 toxin and subsequent coating on ELISA plates may also be a significant improvement in the immunoassay for T-2. This is a potentially useful means for recovering T-2 from test samples and coating on plates for assay.

A satisfactory antigenic form of T-2 toxin has not yet been developed. The experience gained in Phase I provides insight into the biochemical properties required of the T-2 conjugate. Preparation of the T-2 formyl-PE and related conjugates discussed above will allow for the presentation of T-2 toxin in an antigenically favorable manner. Coupling of these conjugates to vesicles will enhance the immune response by selectively directing the conjugate to macrophage and other cells of the immune system.

The Phase II tasks and objectives originally proposed will be significantly advanced by completion of Phase I activities and the additional activities proposed above. That is an ELISA assay will have been developed in the course of Phase I and early Phase II effort. This assay will be practically suited for use in a modestly equipped, remote laboratory. Information on the sensitivity and application of the assay will also be known.

The modified ELISA employing vesicle T-2 as the medium for producing antigen-coated plates could provide an easy route for the development of a fluorescent

immunoassay. In this mode standard T-2 and test T-2 would be attached to vesicles and coated. T-2 antibody with a fluorescent label could then be used as the means for an FIA development.

Other objectives and questions posed for Phase II, such as shelf-life of immunoassay reagents and the composition of an assay kit must still be investigated.

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